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# PHYSIOLOGICAL STUDIES WITH REFERENCE TO THE GERMINATION OF CERTAIN FUNGOUS SPORES.

B. M. DUGGAR.

## INTRODUCTORY AND HISTORICAL.

THIS study was entered upon with the view of ascertaining somewhat more definitely than previous researches have indicated what may be some of the special factors which influence germination. As particular lines of inquiry the following may be mentioned as suggestive. In how far does there exist in the spores of fungi an essential physiological difference, whereby some may germinate by the mere absorption of water, while others may require for this germination a food supply from without? Where a food supply is required, does germination require a perfect food or a particular food? May a chemical irritant, or poison, which is not primarily a food substance, thus function as a stimulus? Is it possible by mechanical means, or by a change of conditions, to furnish the necessary stimulus for germination? Considering the broad field thus suggested, it has yet been possible to study but a very limited number of fungi. Moreover, there are numerous minor questions which must be considered in a subsidiary way.

Using the term germination in its broadest sense, it may be well at the outset to notice some of the conditions of food supply or stimulation characterizing germination in general. As a rule, the seed of the phanerogam requires for germination only water, along with suitable conditions of temperature and requisite oxygen supply. Not even does the force of imbibition of the seed coats have any special action in inciting to activity the dormant faculties. In about a dozen plants upon which I have experimented, the uninvested embryo, that is, entirely free from integuments or externally stored food material, is capable of manifesting the first stages of germination in distilled water.

A few phanerogams which have become adapted to a peculiar environment may require a stimulus of this environment. Koch<sup>1</sup> and Heinricher<sup>2</sup> have shown that seeds of Orobanchaceæ germinate only with the presence of a host plant. Heinricher found that the seed usually germinates in spring or autumn, in periods of greatest humidity. He states that the germination of the seed is indirectly a partial indicator of the health of the host — a substance excreted by the latter being evidently the source of the stimulus. What may be the nature of this attractive substance has not been determined. On the other hand, seeds of *Rhinanthus* seem to germinate without such stimulus.

According to Wiesner<sup>3</sup> the seeds of *Viscum album* are known to germinate only in light, and although apparently mature in autumn, they are not to be forced to germination until the following spring. The same was found true of *Loranthus Europaeus*; and, although light is required for germination, the most favorable conditions of the tropical climate of Buitenzorg could not induce this activity without the intervention of the resting period. Wiesner then surmised that the factors concerned are the gradual availability of the reserve food, a phylogenetic light influence, and the effect of the viscous substance of the integuments. If there are other phanerogams growing in peculiar situations for which a particular stimulus is necessary for germination, the matter seems to await study. We cannot appropriately include in this place seeds of some Rosaceae, for example, which germinate better after passing through the digestive tract of birds, or after artificial treatment with acids.

The germination of pollen has been very much studied, and it is surprising that this has yielded so little of special interest relative to particular or peculiar stimuli. It is of special

<sup>1</sup> KOCH, L.: Die Entwicklungsgeschichte der Orobanchen 119. 1887. Heidelberg.

<sup>2</sup> HEINRICHER, E.: Die Keimung von Lathrea. Ber. d. deut. bot. Gesells. 12: 117-132. 1894.

<sup>3</sup> WIESNER, J.: Vergleichende physiologische Studien über die Keimung europäischer und tropischer Arten von *Viscum* und *Loranthus*. Sitzb. d. kaisl. Akad. d. Wissenschaften zu Wien 103: 423-437. 1894.

significance that the pollen of so many species of plants germinate either in sugar solution or in water. According to Molisch<sup>4</sup> many forms germinate best in about 10 per cent. sugar solution, and concentrations of from 15 to 40 per cent. are often required. This might suggest an osmotic stimulus. Nevertheless, there are many forms germinating well in water, and for other forms perhaps the idea of a special stimulus should be further applied to a study of pollen. Molisch indeed found minute quantities of malic acid a stimulus to the germination of Ericaceae; but, in general, my results indicate that it is only a stronger stimulus than pure water, not equal to sugar, for example.

Lidforss<sup>5</sup> has recently made extensive studies with pollen germination in "pure water," and among many plants whose pollen is so adapted may be mentioned Orchidaceae, Salicaceae, and many Liliaceae, Geraniaceae, etc. Following in the path of still earlier workers along that line, Borodin<sup>6</sup> clearly indicated that light has a stimulating effect for the germination of fern spores. Kny<sup>7</sup> and others further determined the necessity of this factor. Heald<sup>8</sup> has more recently shown that this stimulus may find a substitute in high temperatures; and apparently he has also cleared up some previous inconsistencies. In the same paper, Heald has demonstrated the necessity of light for the germination of moss spores. In a nutrient solution containing peptone or sugar, on the other hand, good germination resulted in darkness; and higher temperatures alone had no stimulating

<sup>4</sup> MOLISCH, H.: Zur Physiologie des Pollens mit besonderer Rücksicht auf die chemotropischen Bewegungen der Pollenschläuche. Sitzb. d. kaisl. Akad. d. Wissenschaften zu Wien 102<sup>1</sup>: 423-427. 1893.

<sup>5</sup> LIDFORSS, B.: Weitere Beiträge zur Biologie des Pollens. Jahrb. f. wiss. Bot. 32; 237-312. 1899.

<sup>6</sup> BORODIN, —: Bull. de l'Acad. imp. de St. Petersburg: 433-440 1867. (ref. Heald, l. c.).

<sup>7</sup> KNY, L.: Beiträge zur Entwicklungsgeschichte der Farnkräuter. Jahr. f. wiss. Bot. 8: 1-15. 1877.

<sup>8</sup> HEALD, F. DE F.: Gametophytic regeneration exhibited by mosses and conditions for the germination of cryptogam spores. Inaugural-Dissertation. Leipzig, 1897.

influence, as likewise small quantities of poisonous substances. The further interesting fact was disclosed that the effect of light is not one of photosynthesis. Equisetum spores, moreover according to Sadebeck,<sup>9</sup> germinate well either in light or in darkness.

Notwithstanding the excellent culture methods for the fungi, a study of germination in relation to the stimuli involved has been largely a matter of incidental consideration. A study of growth phenomena, thermal limitations of growth, toxic effect, etc., have furnished some data relative to the stimulus of particular substances. It seems, however, that no summation of the results has been made since the work of DeBary.<sup>10</sup>

#### METHODS.

The most convenient method of observing spore germination is undoubtedly the hanging drop-culture. The principal points to be considered in properly handling the drop-culture, or Van Tieghem cell, where nutrient media are employed, have been recently set forth clearly by Clark.<sup>11</sup> These notes bear repetition to a certain extent. The employment of large rings is desirable, and they should be cemented to the glass slips by a mixture of refined beeswax and pure vaseline. The cover should be cemented to the ring with vaseline. The same character of liquid should be used at the bottom of the cell as employed in the drop.

While the form of cell culture above described is highly accurate for culturable forms in nutrient media, it is by no means accurate in all other cases. When a careful study is to be made of particular stimulants in water, or in a medium not ordinarily causing abundant germination, and the like, recourse should be had to a different method. My experience has been that the

<sup>9</sup> SADEBECK, R.: Ueber die Entwicklungsgeschichte d. Prothallien u. s. w. der Schachtelhalme. Sitzungsber. d. Versammlung deutscher Naturforscher u. Aerzte zur Hamburg, 1876.

<sup>10</sup> DEBARY, A.: Morphologie und Biologie der Pilze, 376-377. 1884.

<sup>11</sup> CLARK, J. F.: Dissociation and toxic effect. Journal of Physical Chemistry 3: 263-316. 1899.

results may be untrustworthy if any volatile or soluble substance besides the medium employed is used in connection with the cultures. As subsequently mentioned, even the purest vaseline may have an effect on sensitive forms. In all cell cultures in which full nutrient media were not employed, I have used a modified method. The cells were used in small Petri dishes. On the bottom of the Petri dish was placed filter paper with holes made for the insertion of the cells, thus securing them against movement. The covers were laid on without vaseline, and only in a few cases with volatile substances was any vaseline placed on the outer rim of the Petri dish. Dishes with ground-glass tops are preferable. All cultures were kept in moist chambers. The one difficulty remains of opening the cultures for examination, but as it is done only once, or at most twice during the continuance of the experiment, it is perhaps a matter of small significance.

All cells, dishes, flasks, etc., used in these experiments were first cleaned with an alkali, then an acid, and finally, after thorough washing in distilled water, steamed for an hour or two before use. Particular care was taken with the covers, which were also boiled in the cleaning materials, and kept soaking either in distilled water, or in some cleaning agent when not in use. Cultures were kept in the warm room at a temperature of about  $25^{\circ}$  C., this temperature being especially commendable on account of ease of examination at the same temperature.

After considerable experience with vegetable decoctions for the growth of fungi, I have generally adopted a decoction of green string beans or of sugar beets as the best culture medium for most readily culturable fungi. Any absolute standard of strength is impossible, but as a working basis, fifty grams of dry matter for each liter of water has been found convenient; thus for green beans, from an average of analyses, three hundred and ninety-two grams would be required per liter.

The chemicals used have been the purest obtainable; and the sugar was recrystallized by the alcohol method, and subsequently

washed with ether. As a standard nutrient-salt solution the following well-known formula was adopted :

Ammonium nitrate	-	-	-	-	-	1.0 gram
Acid potassic phosphate	-	-	-	-	-	0.5 "
Magnesium sulfate	-	-	-	-	-	0.25 "
Iron sulfate	-	-	-	-	-	Trace
Cane sugar	-	-	-	-	-	3-5 grams
Water	-	-	-	-	-	100 <sup>cc</sup>

As a standard salt solution the above formula has been used without the sugar, the osmotic influence being neglected as of little consequence in comparison with the desirability of having equivalent salt constituents. The experiments with the so-called paraffin water resulted from a test of the value of a paraffin lining for flasks in which distilled water was to be used or kept on hand.

It is evident that so far as the fungi are culturable only pure cultures should be used for inoculation purposes. It is desirable, moreover, to avoid old cultures, and cultures which have been exposed to direct sunlight. When possible, I have used spores from cultures five to ten days old.

#### EXPERIMENTAL.

Table I will serve as the basis of some general comparisons with regard to the amount of germination on various nutrient media. In general a perfect food is the best stimulus for the germination of saprophytic forms, but in particular cases special stimuli are necessary. The standard organic solution has for germination purposes less strength than decoctions of plants. Generally speaking, the standard inorganic solution has about the value of sugar, except for the Mucoraceae. There is considerable difference, however, even with related species of fungi.

Of the purely saprophytic fungi studied, *Oedocephalum albidum* is the only one capable of germinating to considerable extent on pure water. This one exception is suggestive in that it is not necessarily a characteristic of saprophytic fungi that the spores do not contain within themselves the nourishment required for germination. Botrytis, though parasitic at times, would further

TABLE I.  
PERCENTAGE OF GERMINATION.

In all cases the cultures were examined 15 hours after the spores were sown, then in most cases 24 and 48 hours later. When germination was not complete at the end of 15 hours there was very rarely germination. The results as given, however, are for the second or third observation.

Spores of	Culture media				
	Water	Bean decoction	Standard nutr. salt sol.	Standard inorg. sol.	Sugar solution
<i>Aspergillus flavus</i> . . . .	0†	100	100	75	75
<i>Sterigmatocystis nigra</i> . . .	0	100	100	3-75	20±
<i>Penicillium glaucum</i> . . . .	0	100	100	20-50	1—
<i>Ædocephalum albidum</i> . . .	100‡	100	100	50-75	100
<i>Botrytis vulgaris</i> . . . . .	100	100	100	100	100
<i>Monilia fructigena</i> . . . . .	75	100	100	100	100
<i>Circinella umbellata</i> . . . .	0	100	0	5	1—
<i>Mucor erectus</i> . . . . .	?§	100	**	***	25±
<i>Mucor racemosus</i> . . . . .	?§	100	100—	?§	80±
<i>Mucor spinosus</i> . . . . .	0	100	100*	0	1—
<i>Phycomyces nitens</i> . . . . .	0†	100	50-100	0	2-10
<i>Chaetocladium Jonesii</i> . . .	0	100	100	0	80±
<i>Coprinus fimetarius</i> . . . . .	0	5-10	0	0	0
<i>Coprinus comatus</i> . . . . .	0	0	0	0	0
<i>Coprinus micaceus</i> . . . . .	0	100	0	0	0
<i>Boletus</i> sp. . . . .	0	0	0	0	0
<i>Ustilago perennans</i> { summer .	1				...
{ autumn .	50-70	100	...	...	100‡
<i>Ustilago avenae</i> { summer .	2-10	...	...	...	50
{ autumn .	50				100‡
<i>Ustilago striiformis</i> . . . . .	0				2±
<i>Urocystis anemones</i> . . . . .	0	0	0	0	0
<i>Uredo</i> (P. graminis on wheat)	5	20	...	...	0
<i>Uredo</i> (P. graminis on rye) .	50-95	20	...	...	50-100
<i>Uromyces caryophyllinus</i> . .	100‡	75	...	...	100‡
<i>Exoascus</i> sp. . . . .	**	**	...	...	**
<i>Ovularia primulina</i> . . . . .	100‡	75	...	...	75±

\* Abnormal.

‡ Nearly.

\*\* Budding.

† Usually.

§ Small.

\*\*\* Some budding.

confirm this view. With the exception of *Coprinus micaceus*, most of the Hymenomycetes employed fail to germinate readily on bean decoction. Brefeld and others have determined that a large number of Coprini, Clavariae, and Tremellineae germinate well upon dung decoctions.

Glycerin, although slow in action,<sup>12</sup> often gives somewhat

<sup>12</sup> DUCLAUX,—: Annales de l'Institut Pasteur 3: 112. —.

TABLE II.  
PERCENTAGE OF GERMINATION.

Spores of	Strength of solution %	Peptone	Strength of solution	Cane sugar	KH <sub>2</sub> PO <sub>4</sub>	NH <sub>4</sub> NO <sub>3</sub>	MgSO <sub>4</sub>	Strength of solution	KNO <sub>3</sub>
<i>Aspergillus flavus</i>	1	100	$\frac{n}{2}$	20	? †	0	15-30	$\frac{2n}{1}$	5-10
	0.1	100	$\frac{n}{10}$	20-75	? †	20	10-20	$\frac{n}{1}$	5-10
	0.01	50 ±	$\frac{n}{100}$	75-100	? †	70-100	5-10	$\frac{n}{2}$	5-10
	0.001	10-25	$\frac{n}{1000}$	50-75	? †	5-30	....	....	....
	....	....	$\frac{n}{10000}$	30-50	....	....	....	....	....
<i>Sterigmatocystis nigra</i>	1	100*	$\frac{n}{2}$	10-50	? †	0	? †		
	0.1	10†	$\frac{n}{10}$	50-100	0	0	0		
	0.01	10	$\frac{n}{100}$	20-50	0	0	0		
	0.001	? †	$\frac{n}{1000}$	5	0	0			
			$\frac{n}{10000}$	5	0				

\* Nearly, when spores are single.

† Very small

‡ At edges of drop.

TABLE III.  
PERCENTAGE OF GERMINATION IN GLYCERIN.

Strength of solution	<i>Aspergillus flavus</i>	<i>Sterigmatocystis nigra</i>	<i>Penicillium glaucum</i>	<i>Eldocephalum albidum</i>	<i>Botrytis vulgaris</i>	<i>Ustilago avenae</i>	<i>Mucor spinosus</i>
$\frac{n}{1}$	75	0	....	100*	75	50-90	0
$\frac{n}{2}$	100*	0	....	100	10-25	100*	0
$\frac{n}{10}$	75-100	0	....	100*	25	100*	0
$\frac{n}{20}$	....	....	20†	....	....	....	....

\* Nearly.

† After two days.

more germination than sugar, yet with *Sterigmatocystis* the case is reversed. In general, the difference in action has seemed to bear no particular relation to the physical properties of the substance, as for example its power of penetrating membranes.

Most of the smut forms gave good germination on bean decoction, although less on pure distilled water than has been reported by observers using ordinary tap water. *Uromyces caryophyllinus* not only germinated less efficiently in bean decoction than in distilled water, but in beet decoction it failed entirely to germinate.

Of the three salts generally used in the standard nutrient salt solution, ammonium nitrate at a particular concentration gives abundant germination with *Aspergillus flavus*, but has no effect upon *Sterigmatocystis*. In general, the neutral salts give a greater stimulation than the one with acid properties. In this connection, reference should be made to the interesting results of Benecke.<sup>13</sup> He considers the presence of potassium absolutely necessary, and that without this metal no germination, or only traces of germination, can occur. Since his cultures were made in flasks, it is perhaps to be asked if he refers to germination (growth) visible to the unaided eye. Not only do some of the mold fungi germinate to considerable extent in solutions of

<sup>13</sup> BENECKE, W.: Die zur Ernährung der Schimmelpilze notwendigen Metalle. Jahrb. f. wiss. Bot. 28:487-530. 1895.

simple substances, but the form of *Botrytis* which I have used germinates within a few hours on large surfaces of pure distilled water. Moreover, the potassium compounds alone are only very slightly stimulating for germination, and I have found no marked increase in germination of *Phycomyces* and *Penicillium* by the addition of potassium nitrate to a solution of sugar. The above remarks are also partially applicable to the conclusions which Molisch<sup>14</sup> has drawn from his most interesting experiments with *Aspergillus* and *Penicillium*. He found no germination without magnesium, saying: "da ohne Magnesium nicht einmal ein Auskeimen der Pilzsporen stattfindet, und dieses Element weder durch die Elemente der alkalischen Erden (Ca, Str, Bar) noch durch die der Zinkgruppe vertreten werden kann." He also applied these results to all "lower" fungi, that is, apparently, to all culturable forms.

Pepsin and asparagin gave almost no germination with *Sterigmatocystis*. The latter substance had also very little effect on *Aspergillus flavus*, while the percentage of germination with the former substance was as high as ninety.

#### EFFECT OF SPECIAL STIMULI ON GERMINATION.

Under the head of special stimuli, or substances which are not normal sources of food supply, are also included, for convenience, certain carbon compounds, as well as the metallic salts and mineral acids.

In this connection an unexpected result was obtained with cultures of *Aspergillus flavus* on distilled water which had been standing in paraffin-lined flasks. Nearly all of the spores on the edge of the drop, or where single, germinated; and a large per cent. of germination occurred throughout the drop. In similar cultures *Sterigmatocystis* gave usually only 10 to 20 per cent. of germination. *Penicillium* and *Phycomyces* were not stimulated.

Ethyl alcohol affords a marked stimulus for the germination of *aspergillus*; germination being more nearly perfect on the edge of the hanging drop, but occurring markedly throughout.

<sup>14</sup> MOLISCH, H.: Die mineralische Nahrung der niederen Pilze. Sitzungsber. d. Kaisl. Akad. d. Wiss. zu Wien 103<sup>1</sup>: 554-574.



Methyl alcohol is slower in action, and eventually less effective. Immersion of spores for short periods of time in injurious concentrations of ethyl alcohol, and subsequently sowing these spores in water cultures, afford no stimulus for germination.

The results with phenol, here only partially given, were unusually variable. More than with any other substance used, difficulty was here experienced from the evaporation and changes in form of the drop within the culture cell, which may partially account for the dissimilarity of results.

Experiments with chloroform were not made in drop culture, but an exposure of half an hour in a saturated atmosphere was found fatal to *Aspergillus* and *Phycomyces*, and without beneficial effect upon *Sterigmatocystis*.

Ether has proved of little consequence as a stimulus, except with *Aspergillus* at the lowest concentrations used. This was hardly to be expected in consideration of its action on the cell activities. It is to be noted that all of the above mentioned substances penetrate membranes immediately.<sup>15</sup>

At the end of fifteen hours there is an almost inappreciable stimulus with camphor, but its effect gradually increases to the third day.

With *Aspergillus flavus* germination in pure water was increased 10 to 20 per cent. when vaseline was used for cementing the covers to the rings.

The above experiments with *Aspergillus* on alcohol, camphor, strychnine, and vaseline were repeated in flask cultures. Again a concentration of  $\frac{n}{500}$  alcohol gave more than 50 per cent. germination; but under these conditions camphor, strychnine, and vaseline gave uniformly little or no germination. This, as well as other experiments, suggested conditions in the hanging drop more favorable for germination than in flask culture.

Besides the results presented in Table V, an interesting fact is noted in connection with hydrochloric acid, as well as later

<sup>15</sup> OVERTON, E.: Ueber die osmotischen Eigenschaften der lebenden Pflanzen- und Tierzelle. Vierteljahrsh. d. Naturf. Ges. in Zurich 40: 1-43. 1895.

TABLE V.  
PERCENTAGE OF GERMINATION AFTER THREE DAYS.

	Strength of solution	HCl	HNO <sub>3</sub>	Acetic acid	Tartaric acid	Oxalic acid	Cu(NO <sub>3</sub> ) <sub>2</sub>	Strength of solution %	CuSO <sub>4</sub>	MnCl <sub>2</sub> + FeSO <sub>4</sub>	ZnSO <sub>4</sub>
<i>Aspergillus flavus</i>	$\frac{n}{10}$	....	....	0	0	0	....	1.0	....	....	0
	$\frac{n}{100}$	0	0	15	5	0	0	0.1	0	3	1
	$\frac{n}{1000}$	1	5-25	20	10	1-5	0	0.01	0-40	10-20	1-5
	$\frac{n}{10000}$	20-30?*	1-2	15	25-50	1-5	?†	0.001	3-5	10-20	0
	$\frac{n}{100000}$	....	0	2-4	....	....	5-10	0.0001	25	30	....
	....	....	....	....	....	....	....	0.00001	3-10	....	....
<i>Sterigmatoctystis nigra</i>	$\frac{n}{10}$	....	....	0	5	0	....	1.0	....	....	0
	$\frac{n}{100}$	1-3	0	10	1	25†	0	0.1	0	0	0
	$\frac{n}{1000}$	3	1-3	10-20	1-5	2-10	0	0.01	0	0	0
	$\frac{n}{10000}$	5-10	0	20	2-3	3-5	0	0.001	15-30	?†	0
	$\frac{n}{100000}$	....	0	2-4	....	....	0	0.0001	0	?†	....
	....	....	....	....	....	....	....	0.00001	0	....	....

\* On edges.

† Very small.

‡ Throughout; 100 per cent. on the edges.

for some other substances. The spores of *Aspergillus flavus* readily fly off from the surface of the drop, and such spores, falling on the cover glass beyond the limits of the medium, but of course moistened by the slight water of evaporation, gave at  $\frac{n}{100}$  and at  $\frac{n}{1000}$  a germination of 50 to 70 per cent.

The salts of the heavy metals have generally caused a slight increase in the germination of *Aspergillus*, but in no case have they acted very strongly.

A repetition of the experiments with nitric acid,  $\text{CuSO}_4$ ,  $\text{Cu}(\text{NO}_3)_2$  and  $\text{FeSO}_4$  were also made in flask cultures at concentrations ranging from  $\frac{n}{100}$  to  $\frac{n}{100,000}$ . Nitric acid of  $\frac{n}{1000}$  and below has in every case given a small percentage of germination. The spores thus germinated show a considerable length of germ tube. Iron has had a similar but weaker effect. So far as could be ascertained from flask cultures, the copper compounds have at most caused a swelling of the spore, and in isolated cases the very slight protusion of a tube.

Although the stimulating action of the organic acids here included, as also the action of alcohol, may be a very different one from that of the inorganic acids, yet this stimulus of the organic acids is in no case a very remarkable one. As to whether or not these organic substances act as peculiar stimuli, or as food substances direct, there is evidently no good clue, for it is not yet possible to draw the line between those concentrations which should be considered stimulating or poisonous and those at which there may be an action merely as food. Clark's results previously mentioned have shown that alcohol at  $\frac{n}{4}$  is the lowest concentration at which any inhibition of the germination of *Aspergillus* in nutrient media occurred.

In general, the action of the organic acids as food substances has been determined by means of the corresponding salts, and further than this we know very little about them. The stimulus given by  $\frac{n}{100}$  or less of acetic acid to both fungi

deserves mention, although the percentage of germination is not great.

Oxalic acid stands quite alone as a substance stimulating *Sterigmatocystis* more than *Aspergillus*, in fact causing its maximum stimulus at a concentration which affords no germination with *Aspergillus*. This effect on *Sterigmatocystis* was so variant and marked that this acid is to be regarded as a peculiar stimulant for that plant.

#### THE INFLUENCE OF CERTAIN PHYSICAL STIMULI.

From some variations in results obtained it became evident that by the form of the drop and the amount of evaporation therefrom, or perhaps by some other physical force closely connected with these conditions, a considerable stimulus was given to germination. When the cultures of *Aspergillus flavus* were prepared at the same temperature at which they were to be incubated, with all possible precautions being observed as to purity of water and cleanliness of cover glasses, there was seldom more than a fraction of 1 per cent. of germination, provided the culture drop retained its original form and dimensions. In cases of some evident change of conditions within the culture, however, whether by slight dispersal of the drop or by a certain amount of evaporation, the percentage of germination was often greatly increased. In special cases the percentage of germination was as high as 90 after an incubation of fifteen hours, this maximum being reached particularly when the spores were free from each other and collected on the periphery of the drop next to the glass.

Many experiments were introduced with the hope of eliminating the single factors which might be involved and of accurately determining the cause of the stimulus. Unfortunately the matter is as yet very inconclusive.

If even very small amounts of some salts were present in the drop, these during evaporation would be concentrated at the periphery, especially at the edge in contact with the glass. This would hardly be sufficient, however, to account for the

germination observed, and such an occurrence of salts could only come from the well-cleaned glass surface.

As a check on the carefully prepared distilled water, cover glasses were moistened over a steam jet, and upon this condensation the spores were sown as before, the results also being parallel to those previously obtained.

The possible stimulus of rapid evaporation gave only negative results in the following experiment: Properly cleaned ground-glass slips were placed on benches (glass rings) in Petri dishes of distilled water. Strips of filter paper which had been soaked for days in acidulated water and then in distilled water were passed over the edges of the slips with the ends reaching into the water. A clear ground-glass surface of nearly an inch was left between the strips, and on this the spores were sown. This arrangement sufficed to keep a constant thin film of moisture over the glass from which evaporation might readily ensue. The covers of the Petri dishes were slightly raised, and these cultures were placed in a fairly dry atmosphere, in ordinary laboratory atmosphere, and in a moist chamber. After twenty-four hours there was from 10 to 20 per cent. of germination, and those cultures in the dry atmosphere gave perhaps less than the other two. Surface tension could hardly be considered a factor, for these fungi remained practically unchanged after a month on the surface of water in flask cultures.

To test the effect of contact and surface tension with the evaporation factor eliminated, well cleaned glass tubes were drawn out into a capillary end to be used as a culture cell. The tube was partially filled with water and the spores inserted. The water was then forced out until the spores reached capillary parts, when the larger end was also closed with water. Such cultures gave very slight germination after two days.

Attempts to increase the surface tension by means of small quantities of oil in the water gave only negative results. Massart<sup>16</sup> found surface tension productive of contact phenomena in

<sup>16</sup> MASSART, J.: La sensibilité tactile chez les organismes inférieures. Journ. de la soc. méd. et nat. de Bruxelles, December 1890.

bacteria, amoebae, flagellates, etc. Busgen<sup>17</sup> found that *Botrytis* reacts to surface tension by the formation of little bundles of branches perpendicular to the touched surface.

Besides the previously mentioned contact experiments, recourse was also had to the clinostat. By shaking, spores were submerged in flasks containing a small amount of distilled water. These flasks were then rotated horizontally so that there was constant movement of the spores and considerable contact with the glass surface. The results were negative. Likewise experiments made with a shock-imparting clinostat, and also with a combination of the rotation and shock, failed to give any positive results.

A small number of experiments was made to test the effect of evaporation caused by a lowering of the vapor tension. To effect this the cells were arranged in the Petri dishes as before. Water was used in the hanging drop above, except in control experiments, and below was placed the salt solution of various strengths. Over solutions of  $\text{MgSO}_4$ , varying in strength from  $\frac{3}{2}n$  to  $\frac{3}{8}n$ , by far the best germination occurred over  $\frac{4}{3}n$ , where drying out was quite gradual.

According to Lesage,<sup>18</sup> who made a number of experiments to determine the dampness of the air in which spores would germinate best, the spores of *Penicillium* germinate well at all of the higher densities, but reached the lower limit between 82 and 84 per cent. humidity. *Aspergillus* germinates so well ordinarily at the higher humidities that the factor of evaporation seems of more significance.

The action of a change of concentration of the medium was tested in a small way. Spores of *Aspergillus flavus* were sown in Erlenmeyer flasks containing respectively 20, 10, and 5 per cent.  $\text{KNO}_3$  in one series, and in another the same strengths of  $\text{MgSO}_4$ . After twenty-four hours about 5 to 10 per cent. of germination had occurred in the  $\text{KNO}_3$  cultures and about 10

<sup>17</sup>BÜSGEN, M.: Ueber einige Eigenschaften der Keimlinge parasitischer Pilze. Bot. Zeit. 51: 53-72. 1893.

<sup>18</sup>LESAGE, P.: Recherches expérimentales sur la germination des spores du *Penicillium glaucum*. Ann. d. sci. nat. Bot. 8: 309-322. 1895.

per cent. in the  $\text{MgSO}_4$ . Water was then added to these cultures until the liquid was reduced tenfold in concentration. A second examination after the lapse of two days showed little or no increase of germination in the original 10 and 20 per cent.  $\text{KNO}_3$ , but an increase to about 25 per cent. in the 5 per cent. solution, and a similar increase in the 5 and 10 per cent.  $\text{MgSO}_4$ . In the culture containing 20 per cent.  $\text{MgSO}_4$ , the increased germination and the amount of growth was greater than in any other.

#### EFFECTS OF TEMPERATURE AND OXYGEN SUPPLY.

As previously mentioned, Heald found that fern spores kept at high temperature were incited to germination as by light, but a longer period of time was required and the response less uniform. In general I have found very little difference between the germination in water of fungi at  $25^\circ \text{C}$ . and at temperatures nearer the maximum. At  $32^\circ \text{C}$ . there is some increase in the germination of *Aspergillus flavus* in hanging drop, but not in flask cultures. The former might well be due to other conditions than to any augmentation from the higher temperature. On nutrient solution *Coprinus fimetarius* was slightly benefited by the same temperature. Such forms as *Coprinus comatus*, *C. micaceus*, *Boletus* sp., and *Urocystis anemones* could not be incited to germination at higher temperatures when no germination occurred at  $25^\circ$ . Botrytis and Phycomyces were both injured at the temperature of  $32^\circ \text{C}$ ., Botrytis failing to germinate on water, and Phycomyces giving a slight growth at the bottom of the liquid. Changes of temperature from  $28^\circ$  to  $32^\circ \text{C}$ . and *vice versa* did not materially affect the germination of *Aspergillus* and *Sterigmatocystis*. After experiments of various kinds with the aecidiospores of *Puccinia graminis*, Eriksson and Henning<sup>19</sup> found that the best results were secured by placing the spores for a time on melting ice, and then sowing them on water. The next best results were obtained when the spores were soaked in water at  $3^\circ \text{C}$ . for three hours, and then sown at room temperature.

<sup>19</sup> ERIKSSON and HENNING: Die Getreideroste, 71. 1896. Stockholm.

The poorest results were secured when the fresh spores were directly sown at the room temperature. Cooling also had a favorable influence upon the uredospores. This excessive cooling is hardly a natural stimulus. It may be regarded perhaps as a substitution stimulus, able effectually to replace some other incitation of the natural environment.

A few experiments were made with reduced oxygen supply, mainly to see if slight variations in this regard would at all vary the results. With cultures at room temperature with an air pressure of 60<sup>mm</sup> there was no noticeable effect on germination, either in water or in nutrient solution. At below 40<sup>mm</sup> of air pressure there was marked retardation, but since such lower pressures were of little concern in these results, the matter was not carried farther.

#### INHIBITION OF GERMINATION BY NUTRIENT SOLUTIONS.

Various authors have made casual reference to the fact that ordinary nutrient solutions may injure the germination of certain fungi normally germinating in water alone. A thorough study of this matter should throw some light upon the conditions necessary for the penetration of the host plant by the parasite. As yet I have had opportunity to make but few experiments in this direction, but an accidental attempt to make a substitute for bean decoction by adding peptone to the standard nutrient salt solution gave some results of interest with certain smuts used. *Ustilago Avenae* and *U. perennans* gave but a small per cent. of germination on any solution containing 1 per cent. of a German preparation of peptone; and the pure peptone solution gave only 1 or 2 per cent. of germination. An American manufacture of peptone did not act as an inhibiting agent; but the pure peptone solution afforded no better germination than distilled water. On the other hand, *Ustilago* was not inhibited by either preparation of peptone.

Certain rust fungi also comport themselves somewhat peculiarly towards nutrient solutions, as seen in Table VI.

TABLE VI.  
PERCENTAGE OF GERMINATION.

	Distilled H <sub>2</sub> O	1% peptone	Beet decoction	Bean decoction	$\frac{n}{10}$ Sugar Solution	$\frac{n}{5}$ Glycerin	$\frac{n}{10}$ NH <sub>4</sub> NO <sub>3</sub>
<i>Puccinia Helianthi</i> (uredospores)	100—	20	25 *		50	10 *	0
<i>Uromyces caryophil- linus</i> (uredospores)	100—	100—	0	75 *	100—	100—	100—*

\* Germ tubes very short and often ill-formed.

DeBary and others have noted an injurious effect of nutrient media upon the formation of zoospores in certain Peronosporae. The effect is to suppress the amount of zoosporic germination, and to develop germ tubes instead. Wuthrich<sup>20</sup> found the same phenomenon characterizing the germination of *Phytophthora infestans* under the influence of small amounts of poisons. From experiments with *Plasmopara viticola*, I have found no germ tube development; but many nutrient media inhibit the germination of the species. Winogradski and Omeliansky<sup>21</sup> have determined that a number of organic compounds act even at considerable dilutions to hinder or prevent the normal action of the nitrite and nitrate bacteria, and often even to sterilize the solution in which it was attempted to grow these organisms. Among these inhibiting substances are peptone and other albuminoids, glycerin, salts of organic acids, and also ammonia.

We are at this time far from a rational conception of the most important problems concerning the relation between host and parasite. The resistance of species and varieties cannot be viewed merely from a histological standpoint, and so far as the problem is capable of solution, a complicated set of factors is to be expected. If peptone and other nutrient media may be injurious to the germination of certain fungi, not only the

<sup>20</sup> WUTHRICH, E.: Ueber die Einwirkung von Metallsalzen auf die Keimfähigkeit der Sporen einiger parasitischen Pilze. Inaugural-Dissertation, Berne, 1892.

<sup>21</sup> WINOGRADSKI und OMELIANSKY: Ueber den Einfluss der organischen Substanzen auf die Arbeit der nitrifizierenden Mikroben. Centrbl. f. Bact. u. Parasitenk. 5<sup>2</sup>:319-343, 371-387, 425-440. 1899.

poisonous excretions of plants, but all excretions may have their rôle to play regarding infection. At any rate, from the point of view of the fungus, a further study of chemotropism, of stimulants to germination, and of the inhibition of germination and growth by injurious substances cannot fail to lead us somewhat farther toward a knowledge of parasitic attack.

#### RESTING STAGES AND DRYING-OUT OF SPORES.

It is well known that seeds of certain phanerogams do not readily germinate on reaching maturity. From the researches of Weisner and others with rather peculiar phanerogams, it is seen that this resting period is not merely due to an absence of the best conditions, but that the element of time, as far as we know, is absolutely essential. Whether we may in some cases substitute for this element of time artificial changes of condition is mostly a matter of conjecture.

The same remarks will hold in a general way for the so-called resting stages of fungi. For the maturity of the spores of the Peronosporaceae and many of the teleutospores of the Uredineae, as examples, a certain resting period is indispensable. From the cytological studies of Wager,<sup>22</sup> it would appear probable that in the case of *Cystopus candidus* the maturity of the oospores, so far as the external appearances are concerned, does not denote the maturity of the zoosporangium with reference to the full quota of nuclei as a basis for the formation of zoospores. It appears that a resting stage must intervene before the final divisions of the nuclei.

Teleutospores of *Puccinia graminis* germinate best when they have been subjected to all the changes of the winter months. Observations on the presence of a certain coloring matter in the walls of such spores led Dietel<sup>23</sup> to attribute to this certain properties for the prevention of the germination, and for protection against unfavorable conditions. DeBary<sup>24</sup> has found that the teleutospores of the previous harvest cannot be brought to germi-

<sup>22</sup> WAGER, H.: Reproduction of *Cystopus candidus*. Ann. Bot. 10: 245-339.

<sup>23</sup> DIETEL, P.: Flora 74: 151. 1891.

<sup>24</sup> DEBARY, A.: l. c.

nation later than August of the following year, and the optimum germination occurs during the spring of the latter year. Eriksson and Henning<sup>25</sup> are of the opinion that a passage through the animal body will not act as a substitute for the dormant period. Such lines of work have not been systematically followed out, and even for the Uredineae, which are very variable in their disposition toward the resting period, the limitations are not sufficiently known. According to Kühn, Brefeld, and others, as previously mentioned, germination of certain smuts in water can only be induced after a period of rest, while immediate germination may result from the addition of food material. My own results also show that the per cent. of germination with *Ustilago Avenae* and *U. perennans* increases considerably from summer to autumn, even though the material is kept in dry condition.

In other experiments, *Sterigmatocystis nigra* kept dry in the laboratory for five years gave no germination, while material one and two years old gave good germination in nutrient media. This age of the material did not act as a stimulus to germination, however, since only a fraction of 1 per cent. germinated when sown on distilled water. After drying out for four days on slide at 25° C. neither *Aspergillus flavus*, *Penicillium glaucum*, nor *Sterigmatocystis nigra* showed any germination as tested by distilled water.

#### SUBMERGENCE OF SPORES.

Ordinarily the spores of such molds as *Aspergillus flavus* and *Sterigmatocystis nigra* float on the surface of solutions; and, depending upon the solution, they may or may not tend to collect at the line of attachment to the glass in drop cultures. The spores of *Phycomyces* and other *Phycomycetes*, as well as many *Hymenomycetes*, however, very readily sink beneath the surface. To test the capacity of *Aspergillus* for submerged germination, spores were sown in bean infusion between the parts of a strip of mica lightly separated. The mica was then dropped into a flask of bean decoction. After two days the removal and examination of the mica showed that all spores were germinated, those

<sup>25</sup> ERIKSSON and HENNING: op. c. 54.

in the middle, however, having developed a germ tube only about ten times the diameter of the spore, while those on the margin were growing luxuriantly. Under pressure submerged spores do not germinate.

Spores of *Aspergillus flavus* were also sown on a layer of agar beneath a considerable layer of the same material, the upper layer being poured on while the first was still soft. Germination readily resulted. Also spores in flask cultures of  $\frac{x}{50}$  alcohol, submerged by shaking, germinated readily.

#### SOME PECULIARITIES OF GERMINATION VERSUS GROWTH.

The Hymenomycetes will doubtless form an interesting field for the study of germination relative to special stimuli. With studies which are yet merely preliminary I have secured only a single positive result of interest, but in many cases failures are likewise suggestive.

Brefeld, in his *Untersuchungen über Schimmelpilze* (part III), records that spores of Gasteromycetes and of Phallus (p. 174) particularly, as well as other members of the fleshy fungi, do not germinate under any conditions tried. On the other hand, *Coprinus stercorearius* (p. 14), *C. lagopus* (p. 99), and *C. ephemeroides* (p. 117) germinate well on any plant decoction, as likewise spores of Clavariæ and Tremellinae (p. 181). *Coprinus ephemerus* (p. 109) is said to germinate once in perhaps ten trials. While I have not been able to germinate *Coprinus comatus* and *Boletus* sp., these forms have been studied only from fresh spores and from spores kept in the laboratory about two months. *Coprinus fimetarius* has given various small percentages of germination in different vegetable decoctions, but otherwise no germination. A species, which unfortunately was not determined while fresh, but later identified as *Coprinus micaceus*, has given little or no germination in all solutions containing no plant decoction. In bean and dung decoction the same material has furnished perfect germination. The question then of interest was to determine if there might exist in the bean decoction a substance stimulating germination but unnecessary for growth; in other words, if we may here

distinguish between a medium for germination and a growth medium. Spores of this fungus, caught with all possible sterilization precautions, were germinated on bean decoctions in flask cultures. About eight hours after the sowing, the liquid was filtered off in a sterile filter, and the collected mass of germinated spores was removed by a needle to a second filter. Here the spores were washed, and finally transferred to a flask of sterile water. In the latter they remained two days, the water being then poured off and the standard nutrient salt solution added. Growth proceeded gradually, and at the end of one month there was a thick mat covering the bottom of the flask, as if with a circular piece of canton flannel. With all of the precautions observed, and by a comparison of the mycelium, this must be taken to justify the belief that we may here deal with a case in which a medium failing to stimulate to germination may yet afford growth. Bean decoction, moreover, is a better growth medium, and it would seem that the stimulus to germination would be a food stimulus. Nevertheless, the addition of peptone to the standard nutrient salt solution also gave no germination, and if the stimulus is that of a food, it must be considered in the class of peculiar foods.

Some other results, scarcely comparable to the above, may, however, be mentioned at this place. Janczewski<sup>26</sup> has determined that *Ascobolus furfuraceus*, a plant growing normally on the dung of herbivorous animals, could only be prepared for germination by being passed through the digestive tract of such animals. White rabbits were the animals used in his experiments. We have here evidently a case in which the spore is immediately capable of germination provided it may be first acted upon chemically or otherwise, so that it is rendered capable of using the stimulus of the medium on which it normally grows.

DeBary<sup>27</sup> also found that *Onygena corvina*, growing on the feathers of birds of prey, seemed to require a particular stimulus

<sup>26</sup>JANCZEWSKI: Morphologische Untersuchungen über *Ascobolus furfuraceus*. Bot. Zeit. 29: 257-262. 1870.

<sup>27</sup>DEBARY, A.: op. c. pp. 376-377.

of its normal environment in order that germination might be effected.

*Coprinus comatus*, *Boletus* sp., and a few other forms failed also to germinate on filtrates or decoctions of the soil in which the plants grew. The soil filtrate cultures were of course swarming with the bacteria which would thrive under such conditions. Equally futile have been the attempts to germinate these spores in the presence of alkaline substances, in a slightly acid medium, or in the presence of a reducing agent. Hartig<sup>28</sup> mentions the germination of *Merulius lacrimans* in the presence of ammonium and other alkaline compounds, after the failure of many other substances.

Brefeld<sup>29</sup> found that *Tilletia caries* fails to germinate in nutrient solution. If already germinated in water and then transferred to nutrient solution the death and bursting of the promycelium soon occurs.

#### DILUTION OF FOOD MATERIALS.

Concerning the minimum food supply necessary for more or less perfect germination there seem to be almost no references in the literature. The concentration, however, at which some substances begin to attract chemotropically, or practically this lower concentration, has been determined by Miyoshi.<sup>30</sup> For example, cane sugar at 0.01 per cent. attracted the hyphae of *Mucor stolonifer*, and ammonium nitrate attracted the same fungus at 0.05 per cent.; while meat extract of 0.005 per cent. was attractive for *Saprolegnia*. On the other hand, Eschenhagen<sup>31</sup> and others have found that germination and growth of the mold fungi may occur at very high concentrations.

Examining horizontal lines in table VII, these experiments are more or less comparable from the point of view of the concentrations of the medium. The standard nutrient salt solution

<sup>28</sup> HARTIG, R.: Der echte Hausschwamm.

<sup>29</sup> BREFELD, O.: Vgl. Unters. a. d. Gesamtgebiete der Mykologie. Part V, p. 152.

<sup>30</sup> MIYOSHI, M.: Ueber Chemotropismus der Pilze. Bot. Zeit. 52: 1-28. 1894.

<sup>31</sup> ESCHENHAGEN, F.: Ueber den Einfluss von Lösungen verschiedener Concentration auf das Wachstum von Schimmelpilzen. Inaug.-Dissertation, Leipzig. 1889.

TABLE VII.

	Bean decoction			Standard nutr.-salt solution		Sugar solution		
	Strength of solution	Per cent. of germination	Remarks	Per cent. of germination	Remarks	Strength of solution	Per cent. of germination	Remarks
Aspergillus flavus	Standard	100		100		$\frac{n}{2}$	20	From 10-30%
						$\frac{n}{10}$	20	" " "
	$\frac{s}{10}$	100		100		$\frac{n}{100}$	75	Drops poor, spreading
	$\frac{s}{100}$	100*		65±		$\frac{n}{1000}$	10-50	Varying from middle to edges
	$\frac{s}{1000}$	60±	From 40-90% in different cultures	?†	3% in middle of drop to 20% on edges	$\frac{n}{10000}$	5-20	" " "
	$\frac{s}{10000}$	20±	From 5-40% in different cultures	?†	1% in middle of drop to 20% on edges			
	$\frac{s}{100000}$	?†		?†	Slight germination on edges only			
Sterigmato-cystis nigra	Standard	100		100		$\frac{n}{2}$	5	Very few in middle
						$\frac{n}{10}$	20-75	Varied from center to edges
	$\frac{s}{10}$	100		100		$\frac{n}{100}$	10-50	" " "
	$\frac{s}{100}$	20±	Varied from 10-50% about 60%			$\frac{n}{1000}$	5-10	" " "
	$\frac{s}{1000}$	3±		2±		$\frac{n}{100000}$	5-10	" " "
	$\frac{s}{10000}$	0		0				
	$\frac{s}{100000}$	0		0				

\* Nearly.

† Very small.

‡ Small.

|| Practically.

contains 5 per cent. of sugar, the beet decoction about 3 per cent., and the  $\frac{n}{10}$  sugar solution 3.4 per cent. It is noticeable that with Aspergillus on bean decoction practically normal germination takes place as low as  $\frac{s}{100}$  (standard solution diluted one

hundred times), and otherwise the coefficient of maximum germination is constantly above  $\frac{s}{100}$ . Parallel with the results cited above, flask cultures were made with the more variable *Aspergillus*. These cultures for the first two media showed at  $s$  and  $\frac{s}{10}$  complete germination, at  $\frac{s}{100}$  very good germination, at  $\frac{s}{1000}$  a very small per cent., and at further dilutions none. Equivalent cultures on sugar solution are interesting,  $\frac{n}{2}$  giving almost no germination;  $\frac{n}{10}$ , 25-40 per cent.;  $\frac{n}{100}$  and  $\frac{n}{1000}$  perhaps about 10 per cent.; and  $\frac{n}{10000}$  a very small percentage. From this it will be seen that a tenfold dilution of the ordinary culture media affords perfect germination, and a dilution below one thousand times gives practically no germination except with very sensitive fungi.

#### INDIVIDUAL VARIATION OF SPORES.

No studies of importance seem to have been made upon the variation in capacity for germination of individual spores produced under similar conditions, or of spores from the same conidiophore or sporangium. Nevertheless, great individual differences exist, and in any medium which is not a strong stimulus for germination, varying percentages of perfect germination will invariably occur, whatever precautions of method may be observed.

#### CAPACITY FOR GERMINATION OF SPORES LONG INCUBATED ON WATER SURFACES.

Spores of *Aspergillus flavus* and *Sterigmatocystis nigra* were sown on distilled water in Erlenmeyer flasks. At the end of 10, 30, and 90 days some of these were transferred to bean decoction. Practically no germination had occurred on the water, and in nutrient solution of the new cultures these spores gave perfect germination. After 125 days, some germination had occurred among spores in masses, but this germination was by

no means general. The ungerminated spores of these fungi were entirely uninjured as to their capacity for germination after this period of incubation.

#### LENGTH OF LIFE OF GERMINATED SPORES IN WATER.

Spores of *Aspergillus flavus* and *Botrytis vulgaris* were sown in weak bean decoction, and after about eight hours, or when all had germinated, they were filtered and thoroughly washed. The masses of spores were then transferred to fresh distilled water and so preserved. The last sowing from these germinated spores was made after eighty days with *Aspergillus*, and at this time all were yet alive. With *Botrytis*, the last inoculation was made after forty days, with the same result. In the cultures in which these spores were tested, general growth alone was not depended upon, but individual germ tubes were located and growth from these directly observed.

#### LENGTH OF LIFE OF SPORES DRIED OUT AFTER GERMINATION.

Spores of *Aspergillus* and *Botrytis* were germinated as in the above experiments, and then dried on filter paper. With *Botrytis* no further growth could be secured from spores thus dried after twenty-four hours, thus in perfect agreement with the results of Nordhausen.<sup>32</sup> On the other hand, inoculations from the *Aspergillus* material and careful marking and observation of individual germinated spores gave a very general new growth after being dried out twenty days. After sixty-five days there was new growth from about half of those transferred, and after one hundred days there was no sign of growth from germinated spores. It would be interesting to compare further the behavior of parasitic and saprophytic forms in this respect. We know in a general way that the germ tubes of parasitic forms die quickly when dried. Indeed in an early paper by Hoffmann<sup>33</sup> it is stated that "Austrocknen im gekeimten Zustande,

<sup>32</sup> NORDHAUSEN, M.: Beiträge zur parasitärer Pilze. Jahrb. f. wiss. Bot. 33: 1-46.

<sup>33</sup> HOFFMANN, H.: Untersuchungen über die Keimung der Pilzsporen. Jahrb. f. wiss. Bot. 2: 267-337. 1860.

also Austrocknung des Keimfadens, für das Weiterwachsen absolut tödlich ist."

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CORNELL UNIVERSITY,  
Ithaca, N. Y.